The Journal of Molecular Diagnostics, Vol. 13, No. 2, March 2011

DOI: 10.1016/j.jmoldx.2010.10.004

Improved Detection of the *KIT* D816V Mutation in Patients with Systemic Mastocytosis Using a Quantitative and Highly Sensitive Real-Time qPCR Assay

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The vast majority of patients with systemic mastocytosis (SM) carry the somatic D816V mutation in the *KIT* gene. The *KIT* D816V mutation is one of the minor criteria for a diagnosis of SM according to the 2008 World Health Organization classification of myeloproliferative neoplasms. In the present study, we present a real-time qPCR assay that allows quantification of as little as 0.003% *KIT* D816V mutation-positive cells. A total of 61 samples from 31 cases of SM were included in the study. We detected the mutation in skin or bone marrow in 95% of the cases of SM. We demonstrate the clinical relevance of the assay by identifying as little as 0.03% mutation-positive cells in bone marrow aspirates from SM patients and calculate the analytical sensitivity of negative samples to determine the reliability of the result. We further demonstrate that this method also detects the *KIT* D816V mutation in peripheral blood in 81% of the mutation-positive cases with SM. The method also allows comparison of mutation-positive and mast cell fractions to determine whether the mutation is present in non-mast cells, a parameter that has recently been reported to be of prognostic importance in patients with indolent SM. Finally, the assay is suitable for use in prospective studies of the *KIT* D816V allele burden as a treatment endpoint in SM. *(J Mol Diagn 2011, 13:180–188; DOI: 10.1016/j.jmoldx.2010.10.004)*

Mastocytosis is a heterogenous group of diseases characterized by the growth and accumulation of neoplastic mast cells.[1] Based on the clinical presentation of the disease, cutaneous mastocytosis (CM) and systemic mastocytosis (SM) have been defined as the two main subtypes of mastocytosis.[1] CM primarily occurs in children, and the mast cell infiltration is confined to the skin.[1] In contrast, almost all adult patients with mastocytosis have SM, which is characterized by involvement of at least one extracutaneous organ and may involve multiple hematopoetic cell lineages.[1,2] In the vast majority of cases with SM, the clonal nature of the disease can be established through demonstration of a somatic A to T missense mutation at position 2447 of the coding sequence in the *KIT* gene.[1,3,4] The resulting substitution of aspartate (D) to valine (V) at position 816 in the kinase domain leads to autoactivation of the KIT receptor tyrosine kinase.[5] Studies with transgenic mice and mast cell lines have suggested that this mutation alone is sufficient to cause SM.[6–8] However, the *KIT* D816V mutation is not specific for mastocytosis, as gastrointestinal stromal tumors, acute myeloid leukemia, and germ cell tumors have also been demonstrated to carry this mutation.[9]

According to the 2008 World Health Organization classification of myeloproliferative neoplasms, the diagnosis of SM requires the presence of either the major criterion (≥15 mast cells in aggregates in an extracutaneous organ) and one of the four minor criteria (>25% mast cells with atypical morphology, codon 816 *KIT* mutation in an extracutaneous organ, mast cell CD2 and/or CD25 expression, and serum tryptase persistently >20 ng/ml) or three minor criteria.[1] In addition to diagnostic importance, the *KIT* D816V mutation is also important in the treatment of SM by causing resistance to Imatinib mesylate (Gleevec).[10] In contrast, the wild-type KIT and several rare KIT transmembrane (F522C) and juxtamembrane (V559G) mutations are reported to be Imatinib sensitive.[11,12]

Six different variants of SM have been defined: indolent SM (ISM), SM with an associated clonal hematological non-mast cell lineage disease (SM-AHNMD), aggressive SM (ASM), mast cell leukemia (MCL), mast cell sarcoma, and extracutaneous mastocytoma.[1] Patients with the indolent variant (ISM) have a life expectancy not significantly different from that of a sex- and age-matched population, but the median survival of SM-AHNMD, ASM, and MCL is significantly reduced.[13] In a small fraction of patients with ISM, the disease will, however, progress into one of the more aggressive variants, and the presence of the KIT D816V mutation in non–mast cell lineages has recently been identified as one of the most powerful independent parameters for predicting this progression of

Accepted for publication October 1, 2010.

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ISM.[4] The KIT D816V mutation allele burden has furthermore been proposed as a relevant and practical treatment endpoint in SM.[14] At present, no single assay for KIT D816V mutation detection has been accepted as a general standard.[14] Instead, three methodologies (RT-PCR + RFLP, PNA-mediated PCR, and allele-specific PCR) for mutation testing, applied with sufficient sensitivity, have been recommended.[9] With the increasing focus on the clinical applications of the KIT D816V mutation in SM, there is a need for a molecular assay that allows quantification of low mutation levels.

In the present study, we present an allele-specific real-time quantitative PCR (qPCR) assay that allows quantification of as little as 0.003% KIT D816V mutation-positive cells. We demonstrate the clinical relevance of the assay by identifying mutation-positive cell fractions as low as 0.03% in bone marrow aspirates from SM patients, and calculate the analytical sensitivity of samples that test mutation negative to determine the reliability of the result. Furthermore, we use the assay to identify SM patients with KIT D816V mutation in non-mast cells by comparing mutation-positive and mast cell fractions, and propose the assay for use in prospective studies of the KIT D816V allele burden as a treatment endpoint in SM.

Materials and Methods

Patients and Samples

A total of 61 samples from 31 patients diagnosed with ISM (n = 18), ASM (n = 2), SM-AHNMD (n = 4), MCL (n = 1), and SM not classified to variant (n = 6), according to the 2008 World Health Organization classification of myeloproliferative neoplasms,[1] were analyzed for the presence of the KIT D816V mutation (Table 1). The patients were characterized to a varying degree because the major goal of the present study was to validate a new mutation detection method, and all available archival material from cases of SM was therefore included.

All patient samples were obtained from the diagnostic biobank at the Department of Pathology, Odense University Hospital, Denmark. The following types of samples were included in the study; mononuclear cells isolated using Ficoll-Paque density-gradient centrifugation from peripheral blood (PBMNCs; n = 35) and bone marrow aspirates (BMMNCs; n = 19); an EDTA-decalcified, FFPE bone marrow biopsy sample (n = 1); a FFPE bone marrow aspiration clot (n = 1); and skin biopsy samples that were snap-frozen in TissueTek OCT compound (n = 5).

A patient sample previously identified to contain approximately 50% D816V mutation-positive KIT alleles using the Sanger dideoxy DNA sequencing technique was used as a positive control in all real-time qPCR experiments. Negative controls included non-mast cell leukemia cell lines (NB4 and K562) and PBMNCs from 20 healthy blood donors.

All experiments were carried out in accordance with the Declaration of Helsinki and the Danish National Committee on Biomedical Research Ethics.

DNA Extraction

DNA extraction was performed using the MagNA Pure LC Instrument (Roche Applied Science, Mannheim, Germany). DNA was extracted from cell lines, PBMNCs, and BMMNCs (10^6 cells) in a volume of 100 μL of elution buffer using the MagNA Pure LC DNA Isolation Kit I. DNA was extracted from FFPE bone marrow (10 μm section) in 200 μL of elution buffer using the MagNA Pure LC DNA Isolation Kit II, after deparaffinization with xylene and overnight proteinase K digestion. Skin biopsy samples (<10 mg) were homogenized using an UltraTurrax rotor-stator homogenizer (n = 2) or proteinase K digestion alone (n = 3) before DNA extraction in 200 μL of elution buffer using the MagNA Pure LC DNA Isolation Kit II.

Primer and Probe Design

Two real-time qPCR assays with a common forward primer 5′-AGAGACTTGGCAGCCAGAAA-3′ and TaqMan probe 5′−6-FAM-TCCTCCTTACTCATGGTCGGATCACA-TAMRA-3′ were designed.

A reverse primer 5′-TTAACCACATAATTAGAAT-CATTCTTGATCA-3′ for a KIT D816V mutation-specific assay was designed with an intended mismatch at the 3′-minus 2 position to reduce the potential cross-reaction with the wild-type allele. A reverse primer 5′-TGCAG-GACTGTCAAGCAGAG-3′ for a control assay, used for data normalization, was designed with the 3′-terminus 51 nucleotides downstream of the mutational site to amplify both D816V mutated and wild-type KIT alleles. This assay served as an internal control to correct for DNA quantity and quality by quantifying the total number of amplifiable KIT gene alleles in a sample.

The sensitivity (limit of detection) and PCR efficiency of the mutation-specific assay was determined from genomic DNA from 20 healthy blood donor PBMNC samples and standard curves generated from a fourfold dilution series of KIT D816V mutation-positive genomic DNA into wild-type NB4 cell line DNA (Figure 1). Control assay standard curves were generated from a fourfold dilution series of wild-type NB4 cell line genomic DNA into water.

It was verified by agarose gel electrophoresis that both assays produced a single discrete fragment of the expected length (data not shown). Sanger dideoxy DNA sequencing confirmed that the amplicons represented the expected KIT fragments. Mutations in the KIT gene other than A2447T resulting in D816V are not detected by the present method.

Real-Time qPCR

Real-time qPCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification plot and standard curve regression analysis was performed using SDS 2.2 software (Applied Biosystems). Real-time qPCR was performed using the TaqMan Universal PCR Master Mix with AmpErase UNG (Applied Biosystems), 300 nm of each of the forward and reverse primer and 200 nm of the TaqMan probe in a total

Table 1. Percentage KIT D816V Mutation–Positive Cells (qPCR % mut.) and Corresponding Assay Sensitivity (qPCR sens.)

Case ID	Sex	Age (years)	Diagnosis (SM variant)	Tissue type	Sample date (month/day/year)	qPCR % mut.	qPCR sens.	% Neoplastic mast cells	DNA sequencing
1	F	38	ISM	PBMNC	1/9/2006	0.04	0.02		wt
				PBMNC	9/9/2008	0.03	0.02		wt
2	F	48	ISM	PBMNC	7/15/2009	Neg.	0.04		wt
				BMMNC	7/15/2009	Neg.	0.06	Neg. (0.010)	wt
3	M	66	ISM	Skin biopsy	6/4/2009	15	7.5*		wt
				PBMNC	6/4/2009	Neg.	0.02		
4	M	56	ISM	PBMNC	9/10/2004	0.10	0.02		wt
				PBMNC	10/31/2007	0.17	0.02		wt
5	M	44	ISM	PBMNC	7/5/2006	0.91	0.01		wt
				PBMNC	12/4/2007	4.8	0.01		wt
6	M	44	ISM	PBMNC	10/28/2005	0.08	0.01		wt
				BMMNC	11/10/2005	0.69	0.05		wt
				BMMNC	6/4/2010	0.12	0.03	0.020	
7	F	59	ISM	PBMNC	4/26/2007	0.42	0.02		wt
8	M	38	ISM	BMMNC	2/9/2009	1.8	0.24		wt
9	F	57	ISM	PBMNC	1/11/2007	0.41	0.03		wt
				BMMNC	2/2/2009	0.63	0.04		wt
				BMMNC	8/27/2009	0.10	0.009		
10	F	66	ISM	PBMNC	9/23/2009	0.45	0.02		
11	F	66	ISM	Skin biopsy	3/25/2010	3.1	0.19		
				PBMNC	4/9/2010	4.6	0.01		
				BMMNC	4/14/2010	4.9	0.02	0.150	
12	F	65	ISM	BMMNC	1/13/2010	0.16	0.02	0.018	
				PBMNC	3/10/2010	0.06	0.02		
13	M	66	ISM	PBMNC	5/31/2005	0.12	0.03		wt
14	F	50	ISM	Skin biopsy	8/13/2009	23	12*		
				BMMNC	9/4/2009	0.16	0.02	0.008	
15	F	60	ISM	PBMNC	12/5/2007	0.06	0.02		wt
				BMMNC	6/26/2009	6.4	0.12		wt
16	F	61	ISM	PBMNC	4/5/2005	9.1	0.01		wt
				PBMNC	9/1/2009	43	0.04		
				BMMNC	9/4/2009	97	0.02	0.100	
17	M	66	ISM	PBMNC	4/21/2005	Neg.	0.04		wt
				BMMNC	7/27/2006	0.09	0.06		wt
				PBMNC	7/27/2006	Neg.	0.17		wt
18	M	58	ISM	BMMNC	9/28/2006	0.03	0.01		wt
				PBMNC	9/28/2006	Neg.	0.05		wt
19	F	69	ASM	PBMNC	3/21/2006	24	0.04		
20	F	68	ASM	PBMNC	8/5/2004	4.4	0.02		wt
				PBMNC	11/5/2007	12	0.03		wt
21	F	80	SM-AHNMD†	BMMNC	3/23/2010	4.0	0.01		
				PBMNC	4/7/2010	7.0	0.01	Neg. (0.004)	
22	M	65	SM-AHNMD‡	PBMNC	3/11/2010	45	0.01		
23	F	71	SM-AHNMD§	PBMNC	7/4/2008	81	0.02		
				Skin biopsy	1/28/2010	8.2	0.05		
24	M	79	SM-AHNMD¶	PBMNC	1/30/2006	46	0.02		D816V
25	M	66	MCL	PBMNC	7/5/2007	Neg.	0.04		wt
26	F	60	Not classified	PBMNC	5/15/2008	1.1	0.02		
				FFPE BM‖	5/20/2008	36	10		D816V
				FFPE BM**	5/20/2008	19	1.5		wt
27	F	46	Not classified	Skin biopsy	7/20/2009	3.5	1.0*		wt
				PBMNC	2/9/2010	0.50	0.02		
				BMMNC	2/25/2010	0.64	0.007	0.018	
28	F	48	Not classified	BMMNC	11/25/2008	6.7	0.02		wt
29	F	53	Not classified	PBMNC	9/15/2006	0.22	0.01		wt
				PBMNC	1/7/2010	0.46	0.02		
				BMMNC	1/7/2010	0.87	0.01	0.008	
30	F	44	Not classified	BMMNC	12/9/2009	22	0.01	0.220	
				PBMNC	1/22/2010	23††	0.02	Neg. (0.006)	
31	M	51	Not classified	BMMNC	4/7/2009	2.9	0.04		wt
				PBMNC	4/7/2009	0.69	0.02	Neg. (0.010)	

M, male; F, female.

The percentage KIT D816V mutation–positive cells (qPCR % mut.) and corresponding assay sensitivity (qPCR sens.) was measured in 61 samples from 31 cases of SM. In samples with no detectable mutation, results are expressed as mutation negative (neg.). A subset of samples were also analyzed for the presence of neoplastic mast cells using flow cytometry and KIT D816V mutation status using DNA sequencing (wt, wild-type, D816V, mutation detected). In samples with no detectable neoplastic mast cells (neg.), the sensitivity of the sample is indicated in parantheses.

*Reduced analytical sensitivity in three skin biopsy samples with DNA extraction according to a previous protocol not including mechanical homogenization.

The AHNMD of the four cases of SM-AHNMD were as follows: JAK2 V617F mutation-positive essential thrombocythaemia (ET),† Myeloproliferative neoplasm, not otherwise specified (MPN, NOS),‡ (MPN, NOS),§ JAK2 V617F mutation-positive polycythaemia vera (PV).¶

‖Aspiration clot.

**EDTA-decalcified biopsy sample.

††Total leukocyte preparation using NH_4Cl buffer for erythrocyte lysis of the same sample contained 37% mutation-positive cells. Likewise, PBMNC versus total leukocyte preparations from a mastocytosis patient not included in the present study contained 0.20% vs. 0.44% mutation-positive cells, respectively.

volume of 25 μL, including 5 μL of DNA solution. Thermocycling conditions consisted of an initial step of UNG incubation at 50°C for 2 minutes, DNA polymerase activation at 95°C for 10 minutes, and PCR with 50 cycles of denaturation at 95°C for 15 seconds followed by annealing and elongation at 60°C for 1 minute. All qPCR experiments included a no template control (water), a mutation-negative control (NB4 cell line), and a

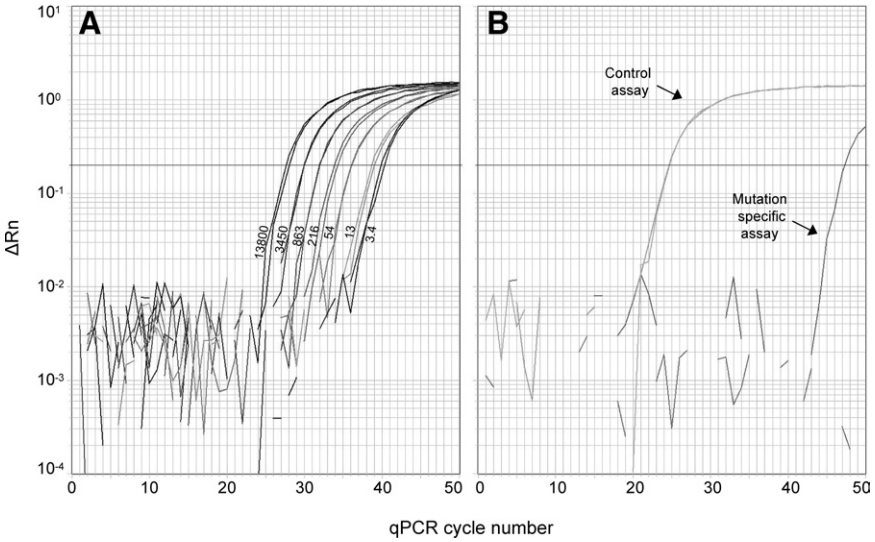

Figure 1. Real-time qPCR amplification plots. **A:** Amplification of a fourfold dilution series of KIT D816V mutation-positive DNA into wild-type DNA by the mutation-specific assay. The undiluted sample contained genomic DNA from approximately 13,800 mutation-positive heterozygous cells. The number of mutation-positive cells in each dilution is indicated at the curve. The average PCR efficiency of four standard curve experiments was 94%. **B:** Amplification of a KIT D816V mutation-negative donor sample by the control and mutation-specific assays. The mutation-specific assay produced amplification in a single replicate as a result of a weak nonspecific cross-reaction with the wild-type allele.

mutation-positive control (patient sample containing approximately 50% mutated alleles) that served as the calibrator sample.

Flow Cytometry

The neoplastic mast cell fraction of three peripheral blood and nine bone marrow aspirate samples was quantified using multiparametric flow cytometry (MFC) immunophenotyping (Table 1). We developed a new six-color MFC assay to increase sensitivity and to reduce the workload of the recommended four-color assay.[9,15] Within 24 hours of their procurement, heparinized peripheral blood or bone marrow suspensions containing at least 10^6 cells were incubated for 15 minutes in the dark, with the following combination of commercially available conjugated monoclonal antibodies in appropriately titrated concentrations: CD45 (2D1-APC-H7), CD117 (104D2-APC), CD33 (P67.6-PerCP-Cy5.5), CD25 (2A3-PE), and CD2 (S5.2-PE-Cy7) from BD Biosciences (San Jose, CA), and CD34 (BIRMA-K3-FITC) from Dako (Glostrup, Denmark). Specimens were lysed and processed using the FACS Lyse Wash Assistant (BD Biosciences), FACS Lysing Solution (BD Biosciences), and CellWASH (BD Biosciences).

Data were acquired and analyzed using a FACSCanto II (BD Biosciences) instrument with FACSDiva 6.1.2 software (BD Biosciences). Mast cell identification was based on the expression levels of CD45, CD117, CD34, and CD33 and their light scatter properties.[15] Aberrant expression of CD25 and/or CD2 identified the neoplastic mast cells.[15] Fluorescence-minus-one controls with all reagents except anti-CD25 or anti–CD2-conjugated antibodies were used to determine the background fluorescence of the mast cells before evaluating the expression level of CD25 or CD2.[16] A cluster of at least 20 mast cells expressing CD25 and/or CD2 was considered positive, resulting in a sensitivity of 0.002% when acquiring 10^6 leukocytes.

DNA Sequencing

A subset of the patient samples included in the study (n = 34) was analyzed for the presence of the KIT D816V mutation using Sanger dideoxy DNA sequencing, in addition to qPCR, to compare the results obtained by the two techniques (Table 1). In DNA sequencing, a thymine peak in addition to the wild-type adenine peak in the chromatograms at position 2447 in the coding sequence of the KIT gene was interpreted as evidence of D816V mutation.

Results

qPCR

The average slope of four standard curve experiments was calculated for both the mutation-specific (−3.47) and control (−3.32) assay, and correspond to PCR efficiencies of 94 and 100%, respectively (Figure 1A).[17] All standard curves produced correlation coefficients >0.99.

The cross-reaction with the wild-type allele of the mutation-specific assay was determined using genomic DNA from 20 healthy blood donor PBMNC samples and two non-mast cell leukemia cell lines (NB4 and K562). In all cases, the threshold cycle (C_t) value was at least 19 units higher in the mutation-specific assay compared with the control assay, corresponding to an assay sensitivity of $1/1.94^{19}$ = 0.0003% (Figure 1B). To be significant regarding detection of D816V mutated KIT alleles, a 10-fold higher cut-off limit, corresponding to 0.003%, was used to define positivity. Furthermore, a mutation-positive sample was defined to have sigmoidic amplification (log scale) and a C_t value of <44. The C_t value of 44 represents the Y-intercept C_t value of the standard curve, which corresponds to the C_t produced in a sample containing one D816V mutated KIT allele + one C_t, in line with the Europe Against Cancer guidelines.[18]

In accordance with the guidelines from the European Study Group on MRD in leukemia, “maximal reproducible

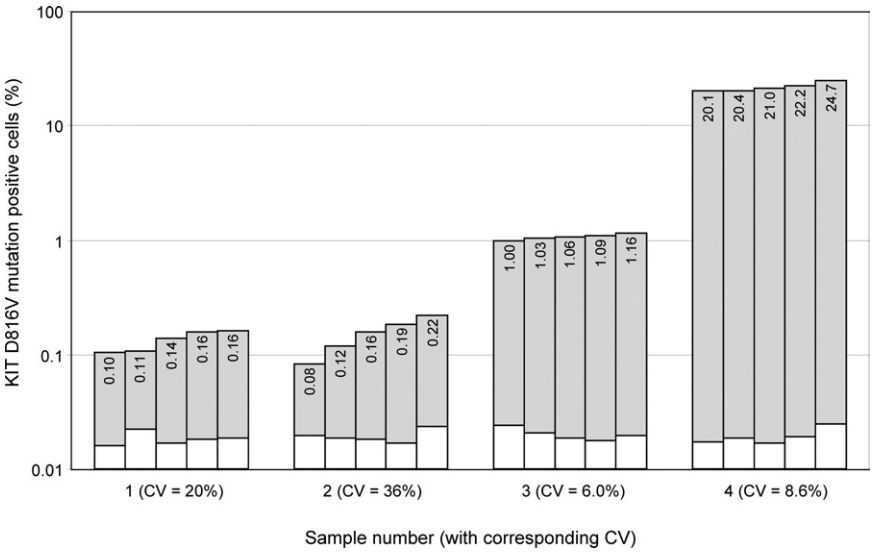

Figure 2. Reproducibility of the KIT D816V mutation detection method determined by repeated analysis of four PBMNC samples in five different qPCR runs. The percentage KIT D816V mutation-positive cells is indicated by a gray bar, with the corresponding assay sensitivity indicated by a white bar.

sensitivity" was defined as the lowest dilution in which all replicates were positive within a 1.5 C_t range.[19] Maximal reproducible sensitivity of the KIT D816V mutation-specific assay corresponded to $C_t = 41$. This C_t detection limit was therefore used to calculate the sensitivity of samples with limited amounts of DNA. Calculation of the KIT D816V mutation-positive cell fraction and assay sensitivity was performed using the following equation[17]:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta Ct,\text{target(control}-\text{sample)}}}{(E_{\text{reference}})^{\Delta Ct,\text{reference(control}-\text{sample)}}}.$$

In the equation, the target represents the mutation-specific assay and the reference represents the control assay. The control represents the calibrator sample containing approximately 50% mutated alleles, which corresponds to 100% mutation-positive heterozygous cells. Results are therefore expressed as the percentage KIT D816V mutation-positive heterozygous cells, which is in line with the general assumption that most tumor cells carry the mutation heterozygously.[20]

The reproducibility of the mutation detection method was determined by repeated analysis of four PBMNC samples with different levels of mutation in five different qPCR runs. The average mutation-positive cell fractions in the four samples were 0.13%, 0.15%, 1.1%, and 22% with corresponding coefficients of variation of 20%, 36%, 6.0%, and 8.6%, respectively (Figure 2). As expected, the level of variation was highest in samples with low levels of mutations. However, no pair of results had a difference of more than 2.8-fold in any of the four samples, and we believe that the reproducibility of the assay is sufficient for the applications proposed in the present study and for clinical applications in general.

The sensitivity of the samples analyzed in the present experiment was below the maximal sensitivity of 0.003% in all cases because of limitations in the amount of DNA. PBMNC, and BMMNC samples typically achieved sensitivities of 0.02% (Table 1), which corresponds to the maximal reproducible sensitivity when qPCR was performed with template from approximately 50,000 cells. DNA extraction from skin biopsy samples homogenized using an UltraTurrax rotor-stator homogenizer produced sensitivities of 0.05% and 0.19% in contrast to 1.0%, 7.5%, and 12% for biopsy samples homogenized using proteinase K digestion alone (Table 1), thus demonstrating an advantage of mechanical disruption of skin biopsy samples before DNA extraction. FFPE bone marrow samples produced relatively low sensitivities (Table 1), most likely as a consequence of the DNA fragmentation and modification occurring during tissue processing.

In addition to the types of samples described above, the mutation has also been detected in RNAlater preserved skin and nonfixed ileum biopsy samples in our diagnostic laboratory from patients not included in the present study, with sensitivities of 0.05% and 0.03%, respectively.

Systemic Mastocytosis Patient Samples

Bone marrow or skin biopsy samples with neoplastic mast cell infiltration were analyzed for the presence of the KIT D816V mutation in a total of 20 cases of SM; ISM (n = 12; Table 1; cases 2, 3, 6, 8, 9, 11, 12, and 14 to 18), SM-AHNMD (n = 2; case 21 and 23), and SM not classified to variant (n = 6; cases 26 to 31).

The mutation was detected in 19 of the 20 cases (95%), with the mutation-positive cell fraction in bone marrow aspirates ranging from 0.03% to 97% (median, 0.9%), and the mutation-positive cell fraction in skin biopsy samples ranging from 3% to 23% (median, 8%).

A single patient with ISM tested mutation negative in BMMNCs with a sensitivity of 0.06%. Using flow cytometry, the sample was determined to contain <0.01% neoplastic mast cells, which is below the limit of detection of the mutation analysis, and we are therefore unable to conclude whether this patient carries the KIT D816V mutation. Alternatively, this patient may carry one of the rare KIT mutations not detected by the qPCR assay.

As expected when using a highly sensitive technique, almost all cases of SM tested KIT D816V mutation posi-

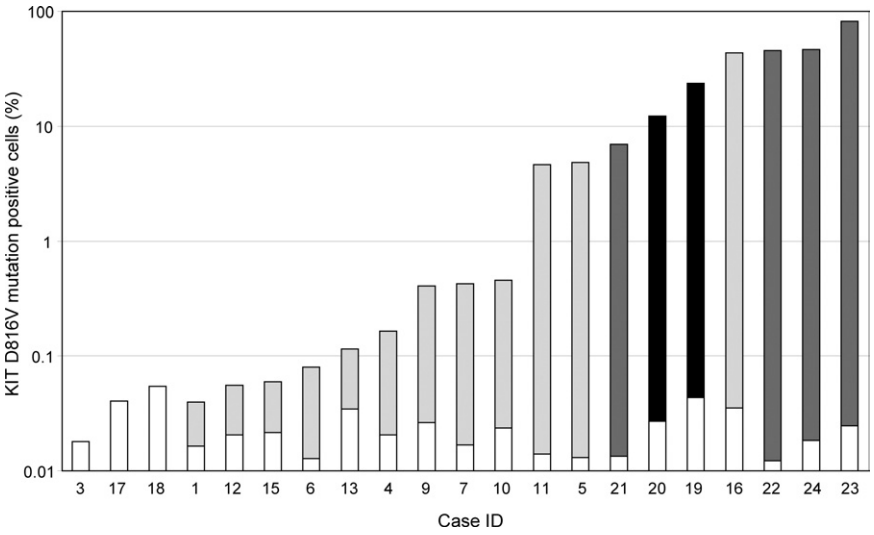

Figure 3. The percentage KIT D816V mutation-positive PBMNCs in two cases of ASM, four cases of SM-AHNMD, and 15 cases of ISM. Case IDs refer to cases in Table 1. In mutation-positive samples, the percentage of mutation-positive cells in cases of ASM is indicated by a black bar, SM-AHNMD by a dark gray bar, and ISM by a light gray bar. The assay sensitivity of the individual samples is indicated by a white bar. Cases 3, 17, and 18, with no detectable KIT D816V mutation in PBMNCs, were all cases of ISM with the mutation detected in tissue, thus confirming that the patients carried the mutation.

tive. Furthermore, mutation-positive cell fractions as low as 0.03% and a median of <1% in BM aspirates clearly underline the importance of using a mutation analysis technique with the highest possible sensitivity to correctly determine the KIT D816V mutational status in unsorted SM patient samples.

In addition to bone marrow or skin biopsies, a PBMNC sample was available in 16 of the 19 mutation-positive cases of SM; ISM (n = 9), SM-AHNMD (n = 2), and SM not classified to variant (n = 5) (Table 1). The KIT D816V mutation was detected in blood in 13 of the 16 patients (81%) with the mutation-positive cell fraction ranging from 0.06% to 81% (median, 0.7%).

The three patients who tested mutation negative in PBMNCs were all cases of ISM with analytical sensitivities ranging from 0.02% to 0.05%. In addition, a single case of MCL was included, and this patient also tested negative for the mutation in a PBMNC sample.

Flow Cytometry

Bone marrow aspirates were analyzed for the presence of neoplastic mast cells in eight cases of mutation-positive SM; ISM (n = 5) and SM not classified to variant (n = 3) (Table 1). Neoplastic mast cells were detected in all cases at levels ranging from 0.008% to 0.2% (median, 0.02%). Peripheral blood was analyzed for the presence of neoplastic mast cells in three cases of mutation-positive SM; SM-AHNMD (n = 1) and SM not classified to variant (n = 2). The fraction of neoplastic mast cells in peripheral blood was below the limit of detection in all cases.

KIT D816V Mutation in Non-Mast Cells

The fraction of mutation-positive cells was quantified in PBMNCs in 21 KIT D816V mutation-positive cases of SM classified to variant [ISM (n = 15; Table 1; cases 1, 3 to 7, 9 to 13, and 15 to 18), ASM (n = 2; cases 19 and 20), and SM-AHNMD (n = 4; cases 21 to 24)]. In 18 of 21 cases, the KIT D816V mutation was detected in peripheral blood with mutation-positive cell fractions ranging from 0.04% to 81% (Figure 3). The presence of the mutation was demonstrated in tissue in the three cases that tested mutation negative in PBMNCs (cases 3, 17, and 18).

Despite the high levels of mutation-positive cells in a number of patients, mast cells were not detected in a peripheral blood smear in any of the 21 patients. Furthermore, peripheral blood was analyzed using flow cytometry in three cases, and the fraction of neoplastic mast cells was below the limit of detection in all cases (<0.01%). With the KIT D816V mutation-positive cell fractions in PBMNC ranging from 7% to 81%, we therefore conclude that all cases of ASM and SM-AHNMD carried the mutation in non–mast cell components. Likewise, the three of the 15 cases of ISM with the highest mutation-positive cell fractions, which ranged from 5% to 43%, were concluded to carry the mutation in non-mast cells (cases 5, 11, and 16).

In five patients, it was possible to quantify the neoplastic mast cell fraction using flow cytometry in addition to the KIT D816V mutation-positive cell fraction in peripheral blood or bone marrow. In three cases that were also determined to carry the mutation in non-mast cells using the approach described above (cases 11, 16, and 21), this more direct approach confirmed the presence of the mutation in non-mast cells with the mutation-positive cell fractions being 33-, 970-, and more than 1750-fold higher than the neoplastic mast cell fractions.

Comparison with DNA Sequencing

A total of 34 samples were analyzed for the KIT D816V mutation using the Sanger dideoxy DNA sequencing technique in addition to real-time qPCR (Table 1). Of the 34 samples, six tested mutation negative with both techniques. Of the remaining 28 samples, all tested mutation positive using qPCR, whereas only two were identified to carry the mutation using DNA sequencing. DNA sequencing thus detected the KIT D816V mutation in only

7% of the samples that were identified to carry the mutation using the real-time qPCR assay. The samples that tested mutation negative using the DNA sequencing method contained up to 19% mutation-positive cells. The samples that tested mutation positive using DNA sequencing contained 36% and 46% mutation-positive cells, thus validating the mutation-positive qPCR results for samples with mutational loads above the detection limit of the DNA sequencing technique. A limit of detection of the DNA sequencing technique between 19% and 36% mutation-positive heterozygous cells is furthermore in good agreement with previous studies reporting detection limits of approximately 20%.[20]

Discussion

In the present study, we present a quantitative and highly sensitive allele-specific real-time qPCR assay for the detection of the KIT D816V mutation, which improves basic mutation testing and facilitates new clinical applications of the mutation in SM.

Determination of the KIT D816V mutational status in SM can be challenging because the degree of mast cell infiltration in affected organs is highly variable, with mast cells often representing a small fraction of the total cell population in patient samples. Using this new qPCR assay, we detected the mutation in skin or bone marrow in 19 of 20 SM patients at levels as low as 0.03% mutation-positive cells in unsorted BMMNCs, thus demonstrating the clinical relevance of the improved sensitivity offered by this assay compared with published methods.[13,20–25] The single case of SM that tested mutation negative in the present study further illustrates the advantage of a quantitative method, in which results from samples that test mutation negative with a limit of detection above the mast cell fraction are readily identified as unreliable.

It was also established that the qPCR method works well on a number of relevant types of patient samples, including mononuclear cells from EDTA-stabilized peripheral blood and heparin-stabilized bone marrow aspirates, FFPE bone marrow, and nonfixed skin biopsy samples. The current diagnostic standard in SM is KIT D816V mutation analysis of unfractionated bone marrow cells (after erythrocyte lysis) or marrow mononuclear cells.[9] In the present study, all samples of peripheral blood and bone marrow aspirate were Ficoll-Paque separated to isolate mononuclear cells. However, with the mutation potentially being present in all hematopoetic lineages, we compared the fraction of mutation-positive cells in preparations of mononuclear cells and total leukocytes (erythrocyte lysis using NH_4Cl buffer) from peripheral blood samples that represented high and low mutation levels. In both patients, the fraction of mutation-positive cells was approximately twofold lower in the PBMNC samples, thus indicating a benefit from the use of DNA from total leukocyte preparations, in line with recommendations on analysis of the JAK2 V617F mutation and BCR-ABL1 fusion gene in related myeloproliferative neoplasms.[26,27]

Validation of a mutation detection technique with improved sensitivity compared with conventional techniques is inherently difficult.[28] However, the mutation-specific qPCR assay produced a very weak cross-reaction with the wild-type allele, and all mutation-positive samples in the present study contained mutation-positive cell fractions at least 100-fold above the highest level of cross-reaction detected in any mutation negative sample. DNA sequencing confirmed the presence of the mutation in samples with mutation levels above the limit of detection of the DNA sequencing technique. The single case of MCL included in the study tested mutation negative, which is in line with reports of the absence of the KIT D816V mutation in a significant number of MCL patients.[29,30]

In a recent study, Escribano et al[4] identified KIT D816V mutation in non-mast cell lineages as one of the most powerful independent parameters for predicting progression from ISM into a more aggressive form of the disease. In SM-AHNMD, however, the presence of KIT D816V mutation in the AHNMD cells appears to be variable and depends on the subtype of AHNMD.[31] A number of approaches for the identification of KIT D816V mutation in non-mast cells have been published, including laser capture microdissection,[31,32] fluorescence-activated cell sorting,[4] and comparison of the fractions of neoplastic mast cells and mutation-positive cells.[20] The latter approach is methodologically simple, as demonstrated in the present study, where the mutation was identified in non-mast cells by comparing the neoplastic mast cell fraction, determined by flow cytometry, and the mutation-positive cell fraction, determined by qPCR. In the majority of the samples included in this retrospective study, the neoplastic mast cell fraction was not quantified. As an alternative to direct comparison of the mutation-positive and mast cell fractions, we therefore used an indirect method to assess mutation status in non-mast cells. A high KIT D816V allele burden in PBMNCs from patients with no mast cells detected in a peripheral blood smear was interpreted as evidence of the mutation in non-mast cells. In agreement with recent studies reporting an association between non-mast cell KIT D816V mutation and aggressive SM variants,[4,20,31] we identified nine cases with mutation in non-mast cells, which included all four cases of SM-AHNMD, the two cases of ASM, and only three of 15 cases of ISM.

Using the new qPCR assay, the KIT D816V mutation was detected in PBMNCs in the majority of cases of SM that also tested mutation positive in skin or bone marrow. Previous studies have also detected the mutation in PBMNCs in a significant proportion (25%) of SM patients.[33] However, in contrast to previous studies in which mutation-positive PBMNC samples were most likely restricted to cases with mutation in non-mast cells, the increased sensitivity of the present method also allowed detection of the mutation in samples with low levels of mutation, thus increasing the clinical applications of mutation testing in blood samples. Pardanani and Tefferi[14] recently advocated for the use of more objective and reproducible treatment endpoints in SM, and proposed further studies examining the KIT D816V allele burden as a disease-relevant and practical endpoint. They also pointed out that no validated and accepted molecular assay is available for the assessment of treatment-in-

duced changes in the allele burden.[14] We believe that the high sensitivity and quantitative nature of the qPCR assay presented in this paper qualifies it as a strong candidate for such an assay. The qPCR technique is furthermore simple and quick to implement and requires the same guidelines and equipment as the molecular minimal residual disease analysis routinely performed in hematological laboratories.[19] Also, the patient group with mutation in non-mast cells typically carries the mutation in a high fraction of cells in peripheral blood. In the present study, these levels were 250- to 4000-fold higher than the detection limit in typical peripheral blood samples. The assay is therefore suitable for further investigation of the KIT D816V mutation level in peripheral blood as a marker of treatment response in this patient group as an analog to the use of molecular markers of minimal residual disease in leukemia.

Acknowledgments

We thank Olav J. Bergmann, Mikael Frederiksen, Henrik Frederiksen, and Sigurd Broesby-Olsen for providing clinical data, and Nina Jensen and Bodil Hansen for technical assistance.

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